An in vitro based investigation biology essay

Science, Biology

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Abstract

In the present study, cytotoxic effects of D-ala, D-pro and D-lys are demonstrated. In an effort to study possible mechanisms of the observed cytotoxicity, Catalase activity, H2O2 generation, and apoptotic activity were measured in HeLa and MCF-7 cell lines. Although D-lys is a poor substrate of DAO and therefore low H2O2 has been detected, it was shown to provoke sever impairment of cellular integrity and survival. Interestingly, a very good substrate of DAO, such as D-pro, did not appear to substantially reduce cell viability. On the other hand, a moderate substrate of DAO, represented by D-ala, has been shown to moderately trigger toxicity in the tested cell lines. Although a correlation between the in vitro cytotoxicity of D-amino acids and the amount of H2O2 produced was absent, there was a good agreement between the ability of D-amino acids to trigger apoptosis and to provoke toxicity. Our results indicate that the toxicity of D-amino acids does not appear to be solely mediated by H2O2. Therefore, we hypothesize that other

possible contributing apoptosis-mediated pathways might cause the observed toxicity.

Introduction

Numerous innovations in analytical methods have demonstrated the presence of D-amino acids in eukaryotes, including mammals (1-3). The most abundant D-amino acids in mammals are D-serine and D-aspartate (4). D-alanine is also present in mammals at moderate levels (4, 5). Recently, the physiological roles of these D-amino acids, in human, have been reported. For example, D-serine has been found to modulate the activity of N-methyl-D-aspartate (NMDA) receptor in the brain (6). It is postulated that D-serine binds to the coagonist binding site of NMDA receptor, which is involved in numerous physiological and pathological processes, including synaptic plasticity, learning, memory, neuronal cell migration, and neural diseases (7, 8). Furthermore, D-aspartate has been detected in nervous and endocrine tissues and is believed to be involved in the regulation of hormone release (9, 10), melatonin (11) and testosterone syntheses (12, 13). Additionally, the presence of free D-aspartate in the nuclei of mammalian tissues may suggest a role of D-aspartate in gene expression (14). In mammals, D-amino acid oxidase (DAO) and D-aspartate oxidase (DAspO) catalyze the oxidative deamination of D-amino acids into the corresponding α-keto acids. D-amino acid oxidase was the first enzyme discovered in mammals (15). It has been found that DAO contains flavin adenine dinucleotide (FAD), as a prosthetic group, and catalyzes the oxidative deamination of D-amino acids according to the reaction mechanism shown in Figure 1. During the initial step of catalysis, oxidation of D-amino acid into the imino acid and concomitant

reduction of FAD occur. Subsequently, the reduced FAD is oxidized by oxygen to release hydrogen peroxide, and the imino acid is non-enzymatically hydrolyzed to produce the corresponding 2-oxo acid and ammonia (16, 17). DAO has a wide range of substrate specificity and metabolizes large number of neutral and basic D-amino acids. Similarly, D-aspartate oxidase has FAD, as a prosthetic group, and catalyzes the oxidative deamination of acidic D-amino acids, such as d-aspartate and d-glutamate, to produce the corresponding 2-oxo acid, hydrogen peroxide and ammonia (16, 17). A major characteristic of DAO and DAspO is their high specificity towards the D-isomers of amino acids; they are almost inactive towards the corresponding L-isomers. Broad substrate specificity of DAO is a plus when considering the overall concentration of the D-isomer of amino acids in food and drinks. The D-isomers of proline, methionine, isoleucine, alanine, valine and phenylalanine are good substrates for DAO (18, 19). Nevertheless, D-ornithine, cis-4-hydroxy-D-proline, N-acetyl-D-ala and D-lys are oxidized at very low rates by DAO (20). Although potential toxicity has been linked to D-amino acids, the fundamental mechanism by which toxicity occurs is not fully understood. In animal studies, administration of D-amino acids to rats and chicks resulted in growth inhibition. In addition, D-amino acids accumulation in certain tissues caused serious damage such as suppression of the synthesis of glutamate oxaloacetate transaminase, glutamic pyruvic transaminase, and lactate dehydrogenase (21). It has been suggested that the toxicity of D-amino acids arises from the oxidative damage to cells by H_2O_2 formed upon the oxidation of D-amino acids (22). Supporting this suggestion, H_2O_2 appears to readily cross cellular

membranes and cause oxidative damage to DNA, proteins and lipids (23-25). Furthermore, H_2O_2 has been found to induce apoptosis of tumor cells in vitro via activation of the Caspase cascade (26, 27). In this study, to assess the in vitro cytotoxicity of D-amino acids, and to gain further insights into the molecular mechanisms that provoke the observed cytotoxicity, we investigated the cytototoxic effects and possible mechanisms of cytoxicity of three D-amino acids; D-alanine, D-proline and D-lysine, using several cell line models.

Materials and Methods

Mammalian Cell Lines and Cell Culture

The cell lines under investigation were human breast adenocarcinoma MCF7, MDA-MB-231 and MDA-MB-453 cell lines, the human ductal breast epithelial tumor cell line T47D, the human pancreatic carcinoma MIA PaCa-2 cell line, the T-cell leukemia HPB-MLT cell line, the human Burkitt's lymphoma Raji cell line, the EBV-negative Burkitt's lymphoma BJAB cell line, the human colon adenocarcinoma Caco-2 cell line, and the human epithelial carcinoma HeLa cell line. Cell lines were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% Heat Inactivated Fetal Bovine Serum (HI-FBS) (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Cell lines were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity. The cells were passaged weekly, and the culture medium was changed twice a week. According to their growth profiles, the optimal plating densities were determined. To ensure exponential growth throughout the experimental period, as well as a linear relationship between absorbance at 490 nm and cell number when

analyzed by the MTS assay, the following densities were used for each cell line: 1×10^4 cells/well for the MCF-7, MDA-MB-231, MDA-MB-453, T47D, MIA PaCa-2, Caco-2, and HeLa cell lines, and 2×10^4 cells/well for the HPB-MLT, BJAB and Raji cell lines.

D-amino acids Cytotoxicity in MCF-7, MDA-MB-231, MDA-MB-453, T47D, MIA PaCa-2, Caco-2, and HeLa Cell Lines

For the assay, cells were washed three times with Phosphate Buffered Saline (PBS) then PBS was decanted and cells were detached with the Non-Enzymatic Cell Dissociation Complex (Sigma Chemical Co., USA). Medium was added to a volume of 10 ml. The cell suspension was centrifuged at 1000 X g at 4°C for 10 minutes and the pellet was re-suspended in 10 ml of medium to make a single cell suspension. Cells were counted using trypan blue exclusion method and seeded into 96-well plates at the desired densities. 100 µL per well of cell suspension was seeded and incubated to allow for cell attachment. After 24 h, the cells were treated with D-amino acids. Each D-amino acid solution at a concentration of 1 M was prepared in BPS and passed through a 0. 02 µm filter then further diluted in the medium to produce the desired concentration. Cells were treated with various concentrations of each D-amino acid in four triplicates. Treated cells were incubated in a 37°C 5% CO_2 incubator for 24 h or 48 h. At the end of the exposure time, MTS assays were carried out using CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's protocol (Promega Co, USA). The absorbance at 490 nm was read on a Tecan Plate Reader (Tecan Group Ltd., Mannedorf, Switzerland). Control wells without D-

amino acids treatment were prepared under the same experimental conditions.

D-amino acids Cytotoxicity in HPB-MLT, BJAB and Raji Cell Lines

Viable cells were counted and diluted with medium to give a final density of 2×10^4 cells/well. 100 µL per well of cell suspension was seeded into 96-well plates. After 24 h, the cells were treated with the D-amino acids. Cells were treated with various concentrations of each D-amino acid in four triplicates. The plates were incubated for 24 h or 48 h at 37°C 5% CO_2. Cell viability was measured using MTS assay as previously described. Control wells without D-amino acids treatment were prepared under the same experimental conditions.

Cell Lysates Preparation

For each assay, HeLa and MCF-7 cells were grown in six-well plates. When the cells reached 80% confluence, they were incubated with either 50 mM D-ala, 50 mM D-pro or 10 mM D-lys for 24 h at 37°C in 95% air 5% CO_2. At the end of the treatment, cells were harvested with Non-Enzymatic Cell Dissociation Complex (Sigma Chemical Co., USA) before centrifuged at 1000 X g at 4°C for 5 min. The pellets were re-suspended in 2 ml of solution consisting of 50 mM phosphate buffer (pH 7. 0) then lysed by several freezing-thawing cycles. The lysates were centrifuged at 3000 X g at 4°C for 5 min, and the supernatants were frozen at -20°C until used. The protein concentrations of the supernatants were determined with the Bio-Rad Protein Assay (Bio-Rad Inc., USA) using bovine serum albumin (BSA) as a standard.

Catalase Assay

Catalase activity was determined as described previously by Aebi (28). The working solution was prepared by adding H_2O_2 to phosphate buffer: First, 50 mM phosphate buffer (pH 7. 0) and 30 mM H_2O_2 solutions were prepared. Then, 0. 3 ml of 30 mM H_2O_2 solution was added into 100 ml of 50 mM phosphate buffer (pH 7. 0) to yield the working solution. Using phosphate buffer (50 mM, pH 7. 0) as a blank, absorbance of the working solution at 240 nm was measured then adjusted to between 1. 15 and 1. 20 by diluting with buffer or adding more H_2O_2 . When the enzyme assay was undertaken, 0. 4 ml of the cell homogenate was added to 3. 6 ml phosphate buffer (50 mM, pH 7. 0) to yield the assay solution. In the test cuvette, 1 ml of the working solution was added to the assay solution and the decrease of absorbance at 240 nm was recorded over a reaction course of 2 min. In the blank cuvette, 1 ml of phosphate buffer (50 mM, pH 7. 0) was added to 2 ml of the assay solution and the change in absorbance at 240 nm was recorded over a reaction course of 2 min.

H_2O_2 Determination

H_2O_2 released by control cells or by cells (5 x 106 cells) treated with either 50 mM D-ala, 50 mM D-pro or 10 mM D-lys, for 24 h, was measured with Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The reaction mixture that contains 50 µM Amplex® Red reagent and 0. 1 U/mL horseradish peroxidase in Krebs–Ringer phosphate was prepared and incubated at 37°C for 10 minutes. After D-amino acid treatment for 6 h, 100 µL of the reaction mixture was added to each microplate well and incubated at 37°C for 30 min

before absorbance at ~560 nm was read on a Tecan Plate Reader (Tecan Group Ltd., Mannedorf, Switzerland). To correct for background absorbance, for each point, the value derived from the untreated control was subtracted.

Cell Death Detection ELISAplus Assay

The Cell Death Detection ELISAplus Assay (Roche, USA) was used to evaluate the apoptotic activity in the cells after incubation with either 50 mM D-ala, 50 mM D-pro or 10 mM D-lys for a period of 24 h. After treatment, the cells were lysed to release cytoplasmic histone associated-DNA-fragments which indicate an apoptosis activity. Absorbance was read at 405 nm. Higher absorbance correlated to increased apoptosis. Negative controls were obtained from untreated cells under the same experimental conditions.

Results and Discussion

In vitro Cytotoxicity of D-amino Acids

Cytotoxic effects of D-ala, D-pro and D-lys were examined via an in vitro system using several cancerous cell lines as described in the Materials and Methods section. Our choice of D-amino acids was based on their susceptibility to be metabolized by DAO. D-pro represents an optimal substrate of DAO with a high turnover rate (19) while D-ala represents a moderate substrate of DAO with an intermediate turnover rate (19). However, D-lys represents a very poor substrate of DAO with a low turnover rate (19). According to our results, 10 mM D-lys and 50 mM D-ala exhibited remarkable cytotoxic effects when incubated for 24 h (Table I), or 48 h (data not shown), with the cell lines under investigation, whereas 50 mM D-pro exhibited minimal cytotoxic effect under the same experimental conditions

(Table I). Concentration-dependent cytotoxicity was observed when D-ala and D-lys were incubated for 24 h with the cell lines under investigation and thus IC50 values were determined (Table II). To confirm that the toxicity observed with the D-amino acids was specific to the D-enantiomer and not to the L-enantiomer, studies under conditions where cells were treated with various concentrations of L-ala, L-pro, or L-lys were undertaken. Our results indicate that the toxicity is only observed for the D-isomer but not the L-isomer at concentrations upto 150 mM of amino acids (data not shown). Several potentially harmful byproducts of normal cellular metabolism directly influence cellular functions and survival (29). Despite the fact that such harmful byproducts are vital for signal transduction pathways (30) and reduction-oxidation status (29), overproduction of these highly reactive metabolites can initiate lethal chain reactions that ultimately lead to impairment of cellular integrity and survival (29). Although the toxicity of D-amino acids has been recognized, the reason for their toxicity has not been revealed yet. Several mechanisms were proposed to explain the toxicity associated with D-amino acids in eukaryotes. The most broadly recognized pathway of D-amino acids' toxicity in eukaryotes appears to be related to the oxidative damage to cells by H2O2 that is formed upon the oxidation of D-amino acids by DAO and/or DAspO (22). In an effort to study the mechanism of cytotoxicity, the Catalase assay and direct H2O2 determination were undertaken as described below.

Catalase Activity

HeLa and MCF-7 cell lines were selected, as representative cell lines, to investigate the mechanisms of the observed cytotoxicity of D-amino acids.

Around 5 x 106 HeLa cells and MCF-7 cells were incubated in 7 ml media with either 50 mM D-ala, 50 mM D-pro, or 10 mM D-lys individually for 24 h. The cells were washed with PBS and collected for the Catalase assay. The enzyme activity was determined by the reduction of absorbance of cell homogenate over two minutes started after the addition of H_2O_2 working solution. Comparing the specific activities (international unit/protein concentration) of Catalase, it is noticeable that the Catalase activities of HeLa cells and MCF-7 cells are most significantly increased, about 10 fold and 8 fold, respectively, greater than the untreated control, in response to the addition of 50 mM D-ala (Figure 2). Nevertheless, only 2 fold increase in Catalase activity in HeLa cells, and 2. 4 increase in Catalase activity in MCF-7 cells were observed when treated with 50 mM D-pro under the same experimental conditions. In contrast, relative to the untreated controls of each cell line, only 10-15% increase in Catalase activity in both HeLa cells and MCF-7 cells was observed when treated with 10 mM D-lys.

H_2O_2 Determination

To support the H_2O_2-mediated toxicity pathway of D-amino acids, and to establish a clear correlation between the cytotoxic effect of the D-amino acids and their DAO turnover rate, we investigated the cytotoxic effects of various D-amino acids, including a good substrate of DAO with a high turnover rate (19) represented by D-pro, a moderate substrate of DAO with an intermediate turnover rate (19) represented by D-ala and a very modest substrate of DAO with a low turnover rate represented by D-lys. The amount of H_2O_2 released into the culture medium after 6 h treatment was monitored in HeLa and MCF-7 cell lines. After 50 mM D-ala treatment, H_2O_2 release

increased by 13-fold and 10-fold relative to the untreated cells of HeLa and MCF-7 cells, respectively. Nevertheless, H2O2 release at 50 mM D-pro was enhanced by only 2-3-folds relative to the untreated cells. Interestingly, H2O2 release at 10 mM D-lys was almost about the same as that of untreated cells (Figure 3). Our results indicate that D-lys, which is a poor substrate of DAO, and therefore low H2O2 is produced (Figure 3), exhibited the highest toxicity amongst the tested D-amino acids (Tables I and II). On the other hand, D-pro, which has a high DAO turnover rate, and thus high H2O2 is expected to be produced, appears to exhibit minimal toxicity to most cell lines under investigation (Tables I and II). In agreement with this, the Catalase activity measured in HeLa and MCF-7 cell lines increased correspondingly to H2O2 produced after D-amino acids treatment (Figure 2 and 3). Catalase plays a central function in the antioxidant defense of the organism, as H2O2 is degraded to H2O (31). As shown in Figure 2, D-ala treatment resulted in 8-10 fold increase in Catalase activity; nonetheless significant accumulation of H2O2 occurs (Figure 3) which might outstrip the scavenging capacity of Catalase and results in the observed toxicity. However, although the treatment with D-lys did alter neither the Catalase activity nor the amount of H2O2 produced, it resulted in considerable toxicity which is possibly not mediated by H2O2. Interestingly, a good substrate of DAO as D-pro showed only 2-3 fold increase in Catalase activity as well as in the amount of H2O2 released. As indicated by our results, D-pro did not exhibit a noteworthy cytotoxicity when compared to D-ala or D-lys.

Cell Death Detection ELISAplus Assay

In an attempt to explore possible mechanisms of the cytotoxicity observed with D-lys and D-ala, we evaluated the apoptotic activities in HeLa and MCF-7 cells after treatment with the D-amino acids. A Cell Death Detection ELISAplus Assay was used to verify whether the significant decrease in cell growth observed after the treatment with D-amino acids was the result of enhanced apoptosis in HeLa and MCF7 cell lines. Figure 4 shows that the maximum increase in apoptotic activity was observed after the treatment with 10 mM D-lys followed by 50 mM D-ala treatment in both cell lines. In contrast, D-pro treatment did not appear to significantly increase the apoptotic activity in both cell lines. . Interestingly, the apoptotic activities, measured by determination of DNA fragmentation, were in great agreement with the cytotoxic effects exhibited by D-lys and D-ala (Figure 4). Recently, based on several pieces of evidence, it has been suggested that D-amino acids' toxicity is directly related to the formation of D-aminoacyl-tRNA. Under tight regulation of cellular protein syntheses and modifications, D-amino acids are usually not incorporated into proteins' structures. High substrate selectivity towards L-amino acids by aminoacyl-tRNA synthetases largely contributes to the preferential incorporation of L-amino acids in proteins' structures (32-35). However, it was observed that Escherichia coli and Bacillus subtilis tyrosyl-tRNA synthetases can potentially catalyze the formation of D-tyrosyl-tRNATyr to the same extent reached with the L-enantiomer (32, 33). Interestingly, extracts of E. coli, yeast, rabbit reticulocytes, and rat liver were shown to have an enzyme activity capable of accelerating the hydrolysis of the ester linkage of D-Tyr-tRNA to release the

free tRNA and D-tyrosine (36). The fate of D-aminoacyl-tRNA has never been studied previously. However, Dedkova et al. demonstrated that D-methionine and D-phenylalanine charged on aminoacyl-tRNA were incorporated into proteins in vitro (37), suggesting that the toxicity of D-amino acids might arise from the mis-incorporation of D-amino acids in proteins' structures resulting in the formation of functionless proteins. Taken together, as demonstrated by our results, the toxicity of D-amino acids does not appear to be solely mediated by H2O2 as previously suggested. Using several measures, our results show that a correlation between the in vitro cytotoxicity of D-amino acids and the amount of H2O2 produced is absent suggesting the presence of other possible contributing apoptosis-mediated pathways of toxicity. Ongoing studies that are attempting to unravel the correlation between the D-amino acids' toxicity and the formation of mis-folded proteins should shed light on other possible toxicity pathways.

Conclusions

In conclusion, in an attempt to study possible mechanisms of the observed cytotoxicity of D-amino acids, we report here, the in vitro cytotoxic effects of D-amino acids with variable DAO turnover rates. Our results reveal that the oxidative damage, results from H2O2 production by DAO upon the oxidative deamination of D-amino acids, may not be the only mechanism contributing for toxicity. These findings may lend further support to the validity of the suggested hypothesis of D-amino acids mis-incorporation in proteins' structures resulting in the formation of functionless protein; hence, toxicity.